PCT/AU00/01184

10/089180

REC'D 07 NOV 2000

WIPO PCT

AU 00/1184

4

Patent Office
Canberra

I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 3073 for a patent by LUMINIS PTY LTD filed on 24 September 1999.

WITNESS my hand this
Third day of November 2000

LEANNE MYNOTT
TEAM LEADER EXAMINATION
SUPPORT AND SALES

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **Cell Cycle Control**

The invention is described in the following statement:

CELL CYCLE CONTROL

The present invention relates to methods for using cell cycle regulators to improve technology relating to pluripotent, multipotent and differentiated cells. More particularly, the present invention relates to methods for identifying pluripotent cells and partially differentiated cells, to methods for facilitating the maintenance and proliferation of pluripotent cells *in vitro*, and to methods for reprogramming differentiated or partially differentiated cells so that the cells are converted to a less differentiated state, including a state of pluripotency or multipotency. The invention also relates to methods of cell cycle arrest, cell synchronization and selection of pluripotent cells, including those derived by reversion of differentiated or partially differentiated cells. This invention also relates to methods for directing the differentiation of cells, including pluripotent cells and multipotent cells along defined differentiation pathways, and to methods for prolonging the lifespan *in vitro* of pluripotent, multipotent or differentiated cells. Also within the scope of the present invention are cells, embryos and animals produced using the methods referred to above.

In this patent application the term "pluripotent" refers to cells that can contribute substantially to all tissues of the developing embryo and substantially all cells of the extra-embryonic tissues (often termed totipotent cells), as well as cells that can contribute to substantially all tissues of the developing embryo without contributing to extra-embryonic tissues. "Multipotent" refers to partially differentiated cells that are able to differentiate further into more than one terminally differentiated cell type.

"Maintenance of pluripotent cells" is to be understood as the maintenance of such cells *in vitro* in an undifferentiated state. It may also include, but does not always include, the understanding that these cells are immortal.

The cell division cycle is normally composed of four distinct phases, which in typical somatic cells take 18-24 hours to complete. The S-phase represents the period when chromosomal DNA is duplicated, this is then followed by a gap phase (G2) where cells prepare to segregate chromosomes between daughter cells

during M-phase. After completion of M-phase, cells enter a second gap phase, G1, which separates M- from S-phase. G1 is of special significance because it is here that a cell decides to continue dividing or withdraw from the cell cycle.

At the molecular level, the cell cycle is controlled by waves of cyclin-dependent protein kinase (Cdk) activities that are activated only at specific times and which drive the cell cycle transitions by phosphorylation of specific substrates. For activity, each Cdk catalytic subunit requires a cyclin regulatory subunit. Cdks acting at the G1 phase include Cdk2 which is regulated by cyclin E, and Cdk4 and Cdk6 which are regulated by cyclin D activities. Additional levels of control are provided by cyclin-dependent kinase inhibitors (CdkIs), such as p16.

Mitogenic signals, under normal circumstances, dictate whether a cell divides or arrests (Figure 1). The major step in mitogen-dependent control of cell proliferation comes at a point in G1, known as the Restriction Point (R-point), where cells commit to entering S-phase and to another round of cell division. At the molecular level, mitogen-dependent intracellular signalling pathways control this 'proliferative switch' by activation of cyclin-associated Cdk activities, which function primarily by phosphorylating the retinoblastoma tumour suppressor protein (pRb). This then allows for the dissociation of pRb from E2F transcription factors, resulting in derepression of target genes essential for the G1 to S transition. On the other hand, failure to activate cyclin-associated kinase activities will leave hypo-phosphorylated pRb associated with E2F, and exit from the cell cycle will follow (Figure 1). It is worth noting that cyclin E can also influence entry into S phase by mechanisms independent of E2F. Significantly, loss of R-point control is held accountable for a wide range of different tumours, occurring primarily as a consequence of deregulated cell proliferation.

The development of the mouse embryo in the first few days following implantation is a very dynamic period characterised by rapid cell proliferation and differentiation. At the time of implantation at 5.0 days post coitum (dpc) the embryo comprises a central ball of Inner Cell Mass (ICM) cells which are pluripotent (they can give rise to all cell types of the later embryo and adult). These are surrounded by the extraembryonic primitive endoderm and

trophectoderm lineages. Around 5.0dpc the pluripotent cells, referred to from this time as epiblast, commence a period of rapid proliferation which accompanies transition from the ICM ball of cells, to a unicellular layer of pluripotent primitive ectoderm (Figure 2). The entire embryo arises from differentiation of the primitive ectoderm into the three germ layers ectoderm, endoderm and mesoderm during gastrulation which initiates at around 6.5dpc with the appearance of a structure called the primitive streak which forms at the posterior region of the embryo (Figure 2). Pluripotent cells migrate through the streak losing pluripotence and emerge as differentiated germ layer cells. From this time pluripotent cells in the embryo are restricted to the future germ cells.

Immediately following implantation the region of the embryo that will contribute to the adult, the primitive ectoderm, consists of around 30 cells which divide once every 10-12 hours. This rate of cell division is marginally faster than that in the intestinal crypt, the most rapidly cycling cells in the adult. The embryo maintains this rapid rate of cell division in the primitive ectoderm cells for the next 48 hours. Just before gastrulation, the cell cycle of the primitive ectoderm decreases even further to around 6 hours (Figure 2). Indeed some propose the existence of a sub-population of cells within the primitive ectoderm with even faster cell cycle times of 3 to 4 hours. This is a quite remarkable shortening of the cell cycle, especially when compared with the length of most somatic cell cycles, which range from 18-24 hours (Figure 2). Accelerated proliferation within the embryonic epiblast therefore precedes, or coincides with, the differentiation of primitive ectoderm and hence, loss of unlimited differentiation potential associated with pluripotency.

The very rapid expansion of the primitive ectoderm generates sufficient cell numbers to support formation of the three germ layers over a short developmental time-frame. This burst of cell proliferation is considered to be the force which drives gastrulation and can account for the transformation of a single layered embryo into the three germ layers, the definitive ectoderm, mesoderm and endoderm. Cell division in the newly formed mesoderm is not considered to play a significant role in expansion of this layer, nor the endoderm. Cell cycle length in

the newly formed mesodermal cells has been measured at 12 hours and given that a complete mesodermal layer forms within 24 hours of the onset of gastrulation, most of the increase in this germ layer must be contributed from elsewhere, namely the primitive ectoderm.

In the post-gastrulation embryo it appears that all cells have cycles of >12 hours. It is therefore apparent that in the early postimplantation embryo the rate of proliferation firstly increases dramatically just prior to and during gastrulation, but then slows after the cells lose pluripotency in the post-gastrulation embryo, and at some time as they differentiate.

Although changes in proliferative rates during embryogenesis have been described, the cell cycle structure, and molecular mechanisms underlying these events have not been determined.

Murine pluripotent cells can be isolated from the pre-implantation mouse embryo as embryonic stem cells (ES cells). ES cells can be maintained indefinitely in vitro, and can contribute to all adult tissues of the mouse, including germ tissues, when introduced into a host blastocyst. These properties of the ES cell have led to the development of a powerful model system for investigating mechanisms of early embryo development. Importantly they also offer opportunities for the precise genetic manipulation of the embryo and for the production of differentiated cells and adult animals with desired genetic features.

In species other than the mouse, the availability of pluripotent cells would provide similar opportunities for precise genetic manipulation, leading to potential commercial and health advantages in areas such as animal husbandry. In the human, pluripotent cells offer novel approaches based on cell therapy, for the treatment of a number of disease states, including genetic diseases, degenerative diseases, some forms of cancer, and hormonal deficiencies.

However, the successful isolation, maintenance in vitro, genetic manipulation and germ-line transmission of pluripotent cells from species other than mouse has generally been unsuccessful to date. Two of the shortcomings

considered responsible for the inability to transfer mouse pluripotent cell technology to other species are:

1. Inability to identify accurately the ES cell equivalent in species other than mouse.

2. Failure to identify culture conditions that support maintenance of pluripotent cells from species other than mouse in a proliferating undifferentiated state.

There have also been other difficulties associated with pluripotency. In particular there have been difficulties that have restricted the successful reprogramming of differentiated cells so that they revert to a pluripotent, or less differentiated state. In particular, there have been difficulties that have restricted the successful reprogramming of differentiated cells so that they revert to a pluripotent, or less differentiated state. There have also been problems in controlling the differentiation of pluripotent cells along defined differentiation pathways.

There have also been difficulties in maintaining primary or untransformed differentiated cells in culture and *in vivo* for prolonged periods. In particular there are biological mechanisms that limit the number of proliferation rounds that such cells can undergo. This limit is termed the Hayflich limit.

It is an object of the present invention to overcome or at least alleviate one or more of the difficulties or deficiencies associated with the prior art.

Accordingly, in one aspect the present invention provides a method of modifying the cell cycle in pluripotent cells, said method including manipulating the activity of a cell cycle regulatory molecule, including a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor and/or tumour suppressor protein, and molecules displaying similar activities, in said pluripotent cells.

The pluripotent cells may be of any suitable type and may be *in vitro* or *in*

vivo. Preferably, the pluripotent cells are stem cells, epiblast cells, ES cells, EPL cells (as described in International Patent application PCT/AU99/0265, the entire disclosure of which is incorporated herein by reference), primordial germ cells (PGCs) or embryonic carcinoma (EC) cells.

The cyclin may be of any suitable type. Preferably the cyclin is cyclin D or cyclin E, or a molecule exhibiting similar activity, or a functionally active fragment or analogue thereof.

The cyclin-dependent protein kinase may be of any suitable type and includes biochemical activities with similar properties. Preferably the Cdk is Cdk4, Cdk6 or Cdk2, or a molecule exhibiting similar activity, or a functionally active fragment or analogue thereof.

The Cdk inhibitor may be of any suitable type. Preferably the Cdk inhibitor is from the INK, Cip or Kip families of Cdk inhibitors. More preferably the Cdk inhibitor is p27, p57, p16, p15, p18, p19 or p21, or a molecule exhibiting similar activity, or a functionally active fragment or analogue thereof.

The tumour suppressor protein may be of any suitable type. Preferably the activity or phosphorylation state of the tumour suppressor protein is manipulated. Preferably the tumour suppressor protein is Retinoblastoma protein (pRb), p107, or p130, or a molecule exhibiting similar activity, or a functionally active fragment or analogue thereof.

The activity of said cyclin, Cdk, Cdk inhibitor or tumour suppressor protein may be manipulated by any suitable technique. Such techniques include but are not limited to manipulation of expression of said proteins, including manipulation gene expression for example by transformation with expression constructs or antisense technology. Such techniques are well known to those skilled in the art and are described in, for example, Sambrook et al (1989) Molecular cloning, A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, the entire disclosure of which is incorporated herein by reference.

Thus, the applicant has surprisingly found that pluripotent cells have a different cell cycle structure compared to differentiated cells, with a shorter cell cycle where cells spend the majority of time in S phase. ES cells do not express or express very low levels of cyclin D, and express high levels of cyclin E constitutively, throughout the cell cycle, and display a corresponding level of associated Cdk activity. Applicant has also found that defined expression profiles of the INK family of Cdk inhibitors are associated with pluripotency: p27 is found in pluripotent cells; Cdk inhibitors p16 and p19 are not essentially present in pluripotent cells; p21 is not essentially expressed in ES cells, but is found in EPL cells. Expression and activity profiles of all cell cycle regulatory molecules can be used to discriminate between pluripotent and differentiated states, and between states of pluripotency. Furthermore expression and activity of cell cycle regulatory molecules during differentiation is lineage specific; expression profiles are different in mesodermal, ectodermal and endodermal lineages. Applicant has also found that the tumour suppressor Retinoblastoma protein (pRb) is maintained in a hyperphosphorylated (inactive) state in pluripotent cells. Accordingly pRb is unable to interact with the E2F transcription factor, and E2F-activated genes are expressed throughout the cell cycle. Finally applicant has discovered that differentiation of pluripotent cells *in vitro* and *in vivo* is causally or mechanistically linked with loss of these cell cycle characteristics.

The unusual structure of pluripotent cell cycles and their unique mode of molecular regulation have been found to be intimately related to their stem cell state. This intimate association identified in pluripotent cells may be used in a number of ways to establish ES technology and its associated applications in a wide range of species.

Firstly cells with properties similar to the cell cycle characteristics found in pluripotent cells are identified as pluripotent cells. Similarly, pluripotent cells in different states of pluripotency can be identified by differences in their cell cycle characteristics. Furthermore other stem cells, including multipotent cells (such as haemopoietic stem cells and neural stem cells) derived by partial differentiation of pluripotent cells and which are capable of differentiating further into a number of

different cell types, may have some or all of these properties.

Difficulties in maintaining and proliferating pluripotent cells *in vitro* can be overcome by enforcing the features of the cell cycle of pluripotent cells. In particular constitutive cyclin E activity can facilitate the maintenance *in vitro* of pluripotent cells. Maintenance and proliferation of pluripotent cells *in vitro* may also be achieved by enforcing Cdk2 expression, (the cyclin-dependent kinase activity normally regulated by cyclin E), so that Cdk2 activity is constitutive and independent of cyclin E regulation. Methods include upregulation of Cdc25, an activator of Cdk2 activity, and/or down regulation of wee 1-like activity, which down-regulates Cdk2 activity. Manipulation of activities associated with cyclin D may also be used for maintenance and proliferation of pluripotent cells. These manipulations may allow cyclin D activities to substitute for cyclin E activities. Manipulations include constitutive upregulation of cyclin D activity, or the cyclin dependent kinases Cdk4 and/or Cdk6 (the Cdks normally regulated by cyclin D). Up regulation of these Cdks may also be achieved by Cdc25.

Similar difficulties associated with the proliferation and lifespan of multipotent stem cells such as haematopoietic stem cells and neural stem cells *in vitro* may be overcome by some or all of these approaches for manipulating cell cycle regulatory molecules and their activities.

Similar difficulties associated with the limited lifespan (Hayflich limit) of primary or untransformed differentiated cells may also be overcome by some or all of the approaches described herein for manipulating cell cycle regulatory activities. By altering these cell cycle regulatory activities, proliferation of differentiated cells and multipotent cells may be maintained *in vitro* or *in vivo*, and lifespan may be prolonged.

Manipulation of the activities of cell cycle regulatory molecules also allows reprogramming of differentiated or partially differentiated cells to form pluripotent cells. For example, a cyclin E expression construct may be introduced into a differentiated cell, and the nucleus of the transgenic cyclin E cell transferred to or fused with an enucleated pluripotent cell (eg, an ES cell or an oocyte). Using this

technology a pluripotent cell with the genetic properties of the original differentiated cell may be formed.

In a further application useful for reprogramming, there is provided a method for cell cycle arrest and cell synchronisation of pluripotent cells said method including manipulating the activity of a cell cycle regulatory molecule, including a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor and/or tumour suppressor protein, and molecules displaying similar activities, in said pluripotent cells.

More particularly, there is provided a method of selecting pluripotent cells derived by reversion from a differentiated or partially differentiated state, said method including manipulating the activity of a cell cycle regulatory molecule, including a cyclin, Cdk, Cdk inhibitor and\or tumour suppressor protein, and molecules displaying similar activities, in said pluripotent cells.

Cell synchronisation is important in maximising the efficiency of nuclear transfer. The ability to synchronise pluripotent cells relies on knowledge of their special cell cycle structure. In particular pluripotent cells spend the majority of their time in S phase, prohibiting the use of G1/S or S phase inhibition alone, such as Thymidine block or aphidocolin treatment to achieve synchrony. The preferred method for synchronising pluripotent cells involves firstly blocking cell cycle passage at phases other than G1/S or S. For example nocodazole destabilises microtubule assembly, and inhibits cell cycle progression at G2/M or M phases. The cells are then released from nocodazole arrest and simultaneously exposed to S phase or G1/S phase arrest by aphidocolin treatment. The time taken for cells to reach G1/S, S phase blockage is known, based on the novel cell cycle structure of pluripotent cells. Once cells reach this blockage after a known passage of time, they can be released from blockage and they proceed through the cell cycle in synchrony.

The lifespan of pluripotent, multipotent and differentiated cells *in vitro* may be prolonged, and proliferation and maintenance of differentiation status achieved by manipulating the activities and expression of tumour suppressor molecules,

such as pRb, and related activities, p107 and p130. In general cell cycle entry may be promoted by hyperphosphorylation and inactivation of tumour suppressor proteins such as pRb, leading to activation of the E2F family of transcription factors. Inactivation of pRb may be achieved by hyperphosphorylation, or by other approaches that include antisense technology, or gene inactivation. Similarly prolonged lifespan, proliferation and continued maintenance of differentiation status may be achieved by constitutive expression or activity of E2F transcription factors. For example, constitutive E2F activity would occur when E2F is manipulated so that it no longer interacts with nor inhibited by pRb or other tumour suppressors.

Manipulation of upstream regulators of cell cycle regulatory molecules may also be used to achieve maintenance and proliferation of pluripotent cells, and the proliferation and maintenance of differentiation status of multipotent and differentiated cells. For example the proto-oncogenes *myc* and *ras* are known upstream regulators of cyclin E activity. Traditionally research and commercial applications have focussed on reducing the activity of such proto-oncogenes, for applications that include treatment of cancers. It is a novel approach to upregulate these proto-oncogenes for applications such as proliferation and maintenance of pluripotent cells.

Enforcement of cell cycle inhibitor activity, such as Cdk inhibitor activity, may be also used to facilitate maintenance of purified populations of pluripotent cells *in vitro*. In a preferred example, Constitutive p16 activity in pluripotent cells does not inhibit cyclin E/Cdk2 activity. Hence it does not inhibit proliferation of pluripotent cells while cells are in an undifferentiated state. However when differentiation is initiated, cyclin D/Cdk4 and/or cyclin D/Cdk6 activity is inhibited by p16 Cdk inhibitor activity, preventing cell proliferation and initiating cell death. In a preferred example constitutive p16 activity may be achieved by transformation with a constitutive p16 expression construct or any other means. Firstly constitutive p16 activity may be used to maintain purified populations of pluripotent cells. Secondly it can also be used to select for survival and maintenance of pluripotent cells from a mixed population of pluripotent and differentiated cells

expressing p16 constitutively. In a similar manner constitutive Cdk inhibitor activity may also be used to maintain and select for multipotent cells, such as haematopoietic or neural stem cells.

In a further aspect of the present invention there is provided a method of reprogramming differentiated or partially differentiated cells to a less differentiated state, said method including manipulating the activity of a cell cycle regulatory molecule, including a cyclin, a cdk, a cdk inhibitor and/or tumour suppressor protein, and molecules displaying similar activities, in said differentiated or partially differentiated cells.

Cdk inhibitor activity may be exploited to achieve reprogramming of differentiated cells to a less differentiated state, in a manner that is independent of nuclear transfer. A preferred form of this approach relies on a Cdk inhibitor such as p16, and its ability to specifically inhibit cyclin D associated activities, without affecting cyclin E. Hence maintenance and proliferation of pluripotent cells ectopically expressing p16 is unaffected. In differentiated cell populations, including multipotent cell populations, it is probable that spontaneous reversion to a less differentiated state occurs at a low frequency, or rarely. Ectopic p16 expression is achieved in these differentiated cells by techniques that include transformation with expression constructs. Rare individual cells within these populations that revert to a pluripotent state are trapped in their reverted pluripotent state by ectopic p16 activity. Differentiated cells, which rely on cyclin D-associated activities for proliferation, are sensitive to p16 activity, and fail to proliferate, and undergo cell death. Reprogrammed cells may then maintained in their pluripotent state by approaches described in this application. In other forms of this approach, any activity or molecule that regulates the cell cycle, and/or confers a susceptibility to differentiated cells, and an insensitivity to pluripotent cells may be used. For example the proto-oncogene *myc* is an upstream regulator of the cell cycle promoting proliferation in pluripotent cells, but induces apoptosis in some differentiated cells. Hence ectopic *myc* expression could also be used to select for cells reverting to a less differentiated state.

In a further aspect of the invention there is provided a method for

controlling the differentiation of pluripotent cells and multipotent cells along defined differentiated pathways said method including manipulating the activity of a cell cycle regulatory molecule, including a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor and/or tumour suppressor protein, and molecules displaying similar activities, in said pluripotent and multipotent cells.

Applicant has surprisingly found that expression of different cell cycle regulatory molecules and their activities may be mechanistically linked to defined differentiation pathways. Differences in the cell cycle regulatory molecules include different cyclins, different Cdks, different Cdk inhibitors, and different phosphorylation states of tumour suppressor proteins such as pRb.

In a further aspect of the present invention there is provided a method of identifying pluripotent or partially differentiated cells, said method including measuring expression of a cell cycle regulatory molecule, including a cyclin, a Cdk, a Cdk inhibitor and/or a tumour suppressor protein, and molecules displaying similar activities, in said pluripotent cells.

The pluripotent or partially differentiated cells and molecules measured may be as hereinbefore described.

In a preferred form of this aspect of the invention, the method includes measuring a cyclin and/or the phosphorylation state of a tumour suppressor protein such as pRb.

Particularly preferred forms of this aspect of the invention include measuring the expression of one or more, of the following:

(a) cyclin D (an absence or a low level of cyclin D associated activity being characteristic of pluripotent cells;

(b) cyclin E (a high level of constitutive expression activity of cyclin E being characteristic of pluripotent cells);

(c) phosphorylation status of pRb (phosphorylation and therefore inactivity of pRb being a characteristic of pluripotent cells);

(d) INK, Cip or Kip family of Cdk inhibitors, for example:

(i) p27 (expression of p27 is characteristic of pluripotent cells),

(ii) p16 and/or p19 (lack of expression is characteristic of pluripotent cells)

(iii) p21 (expression in some pluripotent cells such as ES cells, but not others, such as EPL cells, and therefore may be used to discriminate between certain pluripotent cell types).

In a further aspect of the present invention there is provided a method of facilitating maintenance and/or promoting proliferation of pluripotent cells, manipulating *in vitro* the activity of a cell cycle regulatory molecule, including a cyclin, a cyclin-dependent protein kinase (Cdk), a Cdk inhibitor, and/or tumour suppressor protein, and molecules displaying similar activities, in said pluripotent cells.

The level of said cyclin, Cdk, Cdk inhibitor or tumour suppressor protein may be manipulated by any suitable technique. Such techniques include but are not limited to manipulation of expression of said proteins. Such techniques are well known to those skilled in the art and are described in, for example, Sambrook et al (1989) Molecular cloning, A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, the entire disclosure of which is incorporated herein by reference.

The pluripotent cells may be of any suitable type and may be *in vitro* or *in vivo*. Preferably, the pluripotent cells are epiblast cells, ES cells, EPL cells (as described in International Patent application PCT/AU99/0265, the entire disclosure of which is incorporated herein by reference), primordial germ cells

(PGCs) or embryonic carcinoma (EC) cells.

In a preferred form of this aspect of the invention cyclin E activity may be manipulated so that it is constitutive. This may be achieved, for example, using a transgene expression construct in a manner known to those skilled in the art. This may include use of a construct including an inducible promoter. Constitutive expressions may also be achieved, for example, using factors capable of regulating endogenous cyclin E expression.

In a is still further aspect of the present invention there is provided cells, embryos and animals, in particular, transgenic cells, embryos and animals, produced using the methods of the present invention.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Figure 1: Cell cycle regulation in normal somatic cells and embryonic cells.

Figure 2: Remodelling of the cell cycle during embryonic development/differentiation.

Figure 3: Flow cytometry analysis and cell cycle structure of pluripotent cells compared with somatic cells.

2N and 4N refers to relative DNA content.

Figure 4: Cyclin-Cdk activities in pluripotent cells.

a) cyclin E-Cdk and cyclin B-Cdk activities

b) cyclin D-Cdk activities

Pluripotent cells were unsynchronised (U), arrested at G1/S, S phase with aphidocolin (+A), or arrested at G2/M with nocodazole (+N).

Cyclin E and cyclin D activities were determined by phosphorylation in vitro of recombinant pRb.

Figure 5: Northern analysis of mRNA isolated from synchronised ES cells.

Figure 6: Western analysis of extracts isolated from synchronised ES cells.

Extracts were from ES cells arrested at G1/S phase and released from arrest.

Figure 7: Synchronisation of ES cells

Panels show DNA content of ES cells determined by flow cytometry. Samples were taken from unsynchronised cells (top left panel), and during arrest with nocodazole, subsequent release, and aphidocolin arrest and release (bottom right panel).

EXAMPLE 1: THE CELL CYCLE PROFILE OF PLURIPOTENT CELLS *IN VITRO* AND EPIBLAST CELLS *IN VIVO* ARE SIMILAR, AND ARE STRIKINGLY DIFFERENT TO THE PROFILE IN DIFFERENTIATED CELLS

To determine how the cell cycle is structured in the epiblast at around the time of gastrulation, we performed flow cytometry on purified populations of epiblast cells derived from time-mated Swiss mouse embryos. Briefly, 6.5dpc embryos were dissected from time-mated Swiss mice, Reichardt's membrane and the extra-embryonic part removed, before the visceral endoderm peeled away from the epiblast by pipetting the embryo up and down with a narrow bore pipette. Each epiblast at this stage of development consists of between 750-1000 cells making it necessary to pool approximately 10 embryos to generate sufficient cell

numbers for flow cytometry analysis. After generating a single cell suspension, cells were then fixed and stained with propidium iodide for flow cytometry analysis on the basis of cellular DNA content (Figure 3). This analysis allows the proportion of time spent in G1, S-phase and G2/M to be determined.

Our analysis of epiblasts have included comparisons with embryonic stem (ES) cells, embryonal carcinoma (EC) cells, mouse embryo fibroblasts (MEFs) and immortalized cell lines such as NIH 3T3, Balbc 3T3 and Swiss 3T3. Results reveal some striking features of epiblast and ES cell cycles relative to cell cycles of differentiated somatic cells. First, it is apparent that pluripotent cells, including those of the epiblast, spend the majority of their time in S-phase (~65%) and short proportions of the cell cycle in G1 (~15%) and G2/M (~20%) phases (Figure 3). In the case of G2 and M-phase cells (which can't be discriminated on the basis of DNA content), we suspect these cells lack a G2 phase as the total G2/M period can be accounted for by the time required for M-phase (mitosis and cytokinesis) alone.

The potential consequences of shortened G1 and G2/M phases could be decreased cell cycle length and loss of regulatory controls such as checkpoints.

EXAMPLE 2: PLURIPOTENCY IS ASSOCIATED WITH ABSENCE OF CYCLIN D EXPRESSION

Characterization of epiblast and ES cell cycles by flow cytometry, demonstrates that pluripotent cells lack fully formed G1 and G2 phases. Because these cells have a truncated G1 phase, and because it is here that proliferative controls are exerted through the R-point, we have focussed on the molecular control of G1.

Cyclin D1 is undetectable throughout the entire embryo in the 6dpc egg cylinder and is first detected in the epiblast in a uniform pattern at 6.5dpc and extends into the primitive streak as gastrulation proceeds. In contrast, cyclin D2 is first expressed in a restricted area adjacent to, or coincident with, the primitive streak and the nascent mesoderm emerging from the streak. Hence, cyclin D2 is

only expressed in the subset of cyclin D1-expressing cells associated with the primitive streak. We conclude that the state of pluripotency in the early embryo is associated with lack of cyclin D expression. First appearance of Cyclin D expression occurs at gastrulation, an event associated with proliferation and differentiation.

To compare cyclin D expression in the early embryo with pluripotent cells *in vitro*, we have performed extensive analysis of Cdk activities in ES cells. We confirm that ES cells also lack D-type kinase activities, as judged by *in vitro* kinase assays using GST-pRb as a substrate. Cyclin D protein and mRNA are also present at low levels or undetectable by Western blot (Figure 4b) and Northern analysis, respectively.

EXAMPLE 3: REGULATION OF THE CELL CYCLE IN PLURIPOTENT CELLS

It is difficult to envisage the existence of a functionally operative R-point in pluripotent cells, since in ES cells *in vitro*, and in embryos before the 6-day egg cylinder stage cyclin D mRNA and protein are absent. Moreover, it is difficult to reconcile how mitogenic signals could control tumour suppressor proteins such as pRb in the absence of downstream targets such as cyclin D. This suggests that control of cell proliferation in pre-gastrulation embryos is not coupled to mitogenic signalling pathways by 'classically' defined mechanisms. The paradox is, that in the absence of cyclin D-Cdk activity, proliferation of primitive ectoderm within the epiblast should be compromised because mechanisms that normally inactivate tumour suppressor proteins (such as pRb, p107) are inoperative. What then, controls the activity of these tumour suppressors in pluripotent cells? It is clear that mitogens such as FGF are required for early pre-implantation development, but how they exert their effect on the cell cycle machinery in the absence of cyclin D is puzzling.

PRB ACTIVITY IN EMBRYONIC CELL CYCLES

We have characterized pRb in pluripotent ES cells, both at the functional and biochemical level. We have evaluated pRb phosphorylation status by

Western blot analysis of ES cell extracts prepared from a nocodazole-aphidicolin synchronization protocol developed in our laboratory. pRb protein levels do not change significantly throughout the cell cycle, but are always found in a slow migrating, hyper-phosphorylated form (data not shown). This suggests that pRb may be inactive and unable to interact with E2F and could explain why the absence of cyclin D is not refractory to the proliferation of pluripotent embryonic cells.

The ability of pRb to inhibit E2F transcription factors was determined by measuring the expression of E2F-activated genes. Northern analysis was conducted on RNA isolated from synchronised ES cells, and probed for cyclin E and RRMP-2 mRNA (RRMP-2 refers to a ribonucleotide reductase). Both these genes are transcribed by E2F transcription factors. mGAP mRA levels remain constant throughout the cell cycle, and was used as a control to show relative RNA loading. Results shown in Figure 5 indicate that these genes are active throughout the cell cycle. Hence E2F transcription factors are active, and pRb inhibition of E2F activity does not occur in ES cells.

The ability of pRb to interact with E2F transcription factors was also tested using gel mobility shift analysis. Our work identifies E2F-4 as being the major E2F DNA binding activity in ES cell nuclear extracts. No E2F-pRb complexes were detected by band shift analysis using supershifting antibodies to probe the composition of protein-DNA complexes (data not shown). Instead, a small fraction of E2F-4 is associated with p107, primarily during G1, although the majority of E2F-4 band-shift activity remains in the 'free' form, capable of activating target genes. Similarly no recruitment of p130 into E2F complexes has so far been observed in these experiments. We conclude from this data that E2F target genes are unlikely to be controlled by pRb or pRb related factors, including p130 and p107.

CYCLIN E AS THE PUTATIVE G1 CDK REGULATOR IN PLURIPOTENT CELLS

To learn more about proliferative control in pluripotent cells, we have investigated cyclin E-dependent Cdks. In synchronized ES cells cyclin E-Cdk is

not only very active, it is promiscuously active throughout the cell cycle (Figure 4a). Levels of cyclin E-Cdk2 pRb kinase activity are of comparable activity in G2/M cells and G1 cells and are significantly higher in magnitude than that found in MEFs and NIH 3T3s. This deviates significantly from the normal pattern of cyclin E-Cdk activity in somatic cells, where cyclin E-Cdk activity increases during G1 and collapses during S-phase.

In support of the continued cyclin E-Cdk activity throughout the cell cycle, cyclin E protein itself was shown to be present throughout the cell cycle in synchronised cells by Western analysis (Figure 6).

In the early embryo, our data derived by immunohistochemistry shows that prior to gastrulation, cyclin E staining is intense in all cells of the epiblast. This is consistent with the observation in ES cells that cyclin E levels are invariant throughout the cell cycle. This contrasts the staining pattern in the surrounding visceral endoderm layer (data not shown) where general staining is less intense and only detected in a fraction of cells (this is probably coincident with G1 and S-phase cells). It is interesting to note, that in the absence of cyclin D-Cdk activity (created by a dominant negative Cdk4), moderately high levels of cyclin E-Cdk activity are sufficient to drive passage through G1. Hence, it is possible that in the absence of D-type Cdk activities, elevated cyclin E-Cdk2 activity could functionally compensate.

EXAMPLE 4: IDENTIFICATION OF PLURIPOTENT AND OTHER STEM CELLS

The successful isolation, maintenance in vitro, genetic manipulation and germ-line transmission of pluripotent cells from species other than mouse has generally been unsuccessful to date. One of the difficulties in obtaining pluripotent cells from other species has been the inability to demonstrate simply and rapidly, that putative pluripotent cells are pluripotent. Generally Oct-4 expression has been identified as a reliable marker for pluripotency. Alkaline phosphatase has also been used, but its reliability as a pluripotent cell marker is questionable since a number of differentiated cells also express AP. Recently some novel markers that distinguish between different types of pluripotent cells

were identified (PCT/AU99/00265). The markers L17 and Psc1 are expressed in ES cells and are down regulated in EPL cells. Conversely K7 is upregulated in EPL cells compared to ES cells.

We have found that expression of cell cycle markers can be determined rapidly in vitro, and cyclin expression data can be used alone, or in combination with other markers to establish the pluripotent state or otherwise of putative pluripotent cell populations. In particular the type of cyclin expressed, and the phosphorylation state of pRb can be used as markers for pluripotency. Absence or low level cyclin D expression, high level constitutive expression of cyclin E, and hyperphosphorylated pRb are used as criteria for pluripotency. Expression of the INK family of Cdk inhibitors also serve as markers for pluripotency. The Cdk inhibitor p27 is found in pluripotent cells, and p16 and p19 are not. p21 is not expressed in ES cells, but is found in EPL cells. Hence p21 can be used to discriminate between states of pluripotency.

These criteria may also be used to identify other stem cells. Many differentiated cells in the mammal are not replaced by proliferation of existing differentiated cells, but by the proliferation of precursor cells (stem cells). Stem cells combine a number of properties that distinguish them from terminally differentiated somatic cells.

They are competent to differentiate into one or more terminally differentiated cell types. For example the haematopoietic stem cells can differentiate into at least 9 different kinds of blood cell.

They are immortal, a property shared only by transformed cells.

They have a capacity for renewal which, if not infinite, extends beyond the lifetime of the animal.

Cellular decisions taken by stem cells are exquisitely regulated by external cues, which link stem cell behaviour to the requirements of the organism. Rates of renewal and differentiation are coupled to prevent depletion of the stem cell

population and control the rate of differentiated cell production, while the pathway of stem cell differentiation can be varied in response to environmental stimuli.

Cell cycle markers characteristic of pluripotent cells are also likely to be useful in identifying other stem cell populations. Although the cell cycles of ES cells are unusually short, this may not be a feature of other stem cell populations driven by cyclin E. For example, haematopoietic stem cells can proliferate slowly, but at the molecular level cell cycle regulation may be similar to ES cells.

EXAMPLE 5: MAINTENANCE AND PROLIFERATION OF PLURIPOTENT CELLS *IN VITRO* BY MANIPULATION OF CYCLIN GENE EXPRESSION

A major problem in obtaining pluripotent cells from species other than mouse has been the inability to proliferate and maintain pluripotent cells in vitro. Manipulation of cyclin activity in putative pluripotent cells offers one approach to overcome these difficulties.

For example constitutive expression of cyclin E from a transgene expression construct would force continuing rounds of cell proliferation by maintaining pRB in a hyperphosphorylated state, and promoting transcription of E2F-regulated genes.

Differentiation of pluripotent cells is associated with a change in the cell cycle from cyclin E-Cdk regulation to regulation by cyclin D-Cdks. Constitutive cyclin E expression from a transgene construct would over-ride cyclin D regulation, and maintain the pluripotent environment.

Constitutive cyclin E activity in pluripotent cells in vitro could be achieved by several approaches, including identification and use of factors regulating endogenous cyclin E gene expression in pluripotent cells, production of transgenic animals, or transformation in vitro of early embryo cells. Transgenic animals would be produced using a cyclin E expression construct, and mated to derive transgenic embryos. Pluripotent cells expressing cyclin E would be isolated from the embryos and cultured in vitro. Alternatively pluripotent cells derived from non-transgenic early embryos or primordial gonad tissue could be transformed in vitro

with a cyclin E expression construct.

Controlled differentiation is central to many of the commercial applications of pluripotent cells. For differentiation of the pluripotent cells transgenic for cyclin E, release from constitutive cyclin E expression is essential. Release could be achieved by use of a cyclin E expression construct that includes Cre-lox recognition sequences. The cyclin E expression construct would be removed from pluripotent cells by application of the Cre-lox system. Alternatively an inducible promoter could be included in the expression construct. Constitutive cyclin E expression is achieved so long as the inducer is included in the culture medium, and when differentiation of the pluripotent cells is required, the inducer would be removed.

EXAMPLE 6: ENFORCED P16 ACTIVITY IN PLURIPOTENT CELLS PROMOTES MAINTENANCE IN VITRO OF PURIFIED PLURIPOTENT CELL POPULATIONS.

An expression construct was prepared containing the p16 gene linked to the EF1α promoter (EF1α is a translation elongation factor and is ubiquitously expressed). The construct also contained an IRES sequence, with the puro^R marker located downstream. Hence both p16 and puro^R are translated from one mRNA. Es cells were transformed with the p16 - puro^R expression construct, and puro^R colonies selected. These puro^R colonies continued to proliferate and maintain their undifferentiated state when cultured in vitro. We concluded that p16 is inactive in pluripotent cells.

In normal ES cell culture a small proportion of the cells near the boundary of the colony differentiate spontaneously, and these differentiated cells are identified readily by their flattened morphology. In the puro^R ES cell colonies expressing p16, a small number of differentiated cells were identified as expected. These differentiated cells failed to proliferate because p16 inhibition of cyclin D. Surprisingly these differentiated, non-proliferating cells died in culture.

These results demonstrate that ectopic expression of p16 in pluripotent cells selects against differentiated cells within the population, and provides a

method for maintaining and proliferating purified populations of pluripotent cells in vitro. This approach can also be used to select for pluripotent cells when in a mixed population of pluripotent and differentiated cells.

EXAMPLE 7: REPROGRAMMING OF SOMATIC CELLS

Technology that allows reversion of differentiated cells to a pluripotent state, or stem cell state has many applications that include cell therapy approaches for the treatment of diseases, and the genetic manipulation of animals. For example, nuclear transfer is a developing technology, where the genetic information from differentiated cells is reprogrammed by transfer into an enucleated oocyte. The resulting cell is pluripotent, and able to undergo normal embryonic development.

Reversion of differentiated cells to pluripotency can also be achieved by approaches that involve manipulation of regulators of the cell cycle. These approaches rely on manipulating cyclin E activity in differentiated cells, so that cyclin E activity is expressed constitutively in the resultant pluripotent cell. For example a differentiated cell is transformed with a cyclin E expression construct and used as a donor cell in nuclear transfer. In one of these approaches, the donor cell is fused with an enucleated pluripotent cell. In an alternative approach the recipient cell is an enucleated oocyte, and the nuclear transfer product is cultured in vitro to allow embryonic development, and the isolation of ES equivalent cells. Both of these approaches allow the production of pluripotent cells with the genetic characteristics of the donor differentiated cell, and where an undifferentiated, proliferative state is maintained in vitro.

Many of the potential uses of pluripotent cells so derived rely on controlled differentiation. Release from constitutive cyclin E expression and an enforced undifferentiated state would be achieved by preventing further constitutive cyclin E expression. For example use of inducible cyclin E expression constructs, or constructs that allow removal of the exogenous cyclin E gene (eg by Cre-lox), as described in the previous example would result in production of a pluripotent cell now permissive for differentiation.

EXAMPLE 8: SYNCHRONISATION OF PLURIPOTENT CELLS

Knowledge of the special cell cycle features of pluripotent cells is essential for synchrony in vitro of pluripotent cells. In particular the cell cycle in pluripotent cells is rapid, and the cells spend the majority (~65%) of time in S phase. This feature prohibits use of S phase and G1/S boundary phase cell cycle inhibitors alone. Such inhibitors include thymidine block, and aphidocolin, which act by arresting cells at any stage from G1/S, through S phase. When pluripotent cells are arrested with such inhibitors, and then released, the cell cycle commences from widely differing time points within phases G1/S through S. Hence cells arrested late in S phase move through G2 and M phases well before other cells arrested at G1/S. Such cells are not synchronised.

Synchronisation of pluripotent cells is achieved by a method that includes sequential arrest at two distinct phases of the cell cycle (Figure 7). Firstly unsynchronised cells were arrested with nocodazole, which destabilises microtubules, and arrests at the G2/M phase, for a time sufficient for all cells within the population to reach arrest. Cells are then released from nocodazole arrest in the presence of aphidocolin. Hence released cells are arrested for a second time at G1/S. Cells then move through S phase in synchrony when aphidocolin is removed. Figure 7 shows flow cytometry analysis of cell samples taken at several time points through the synchronisation procedure, beginning with unsynchronised cells, moving through nocodazole arrest and release, and aphidocolin arrest and release. Cells in the bottom right panel of Figure 7 are synchronised as they move through S phase.

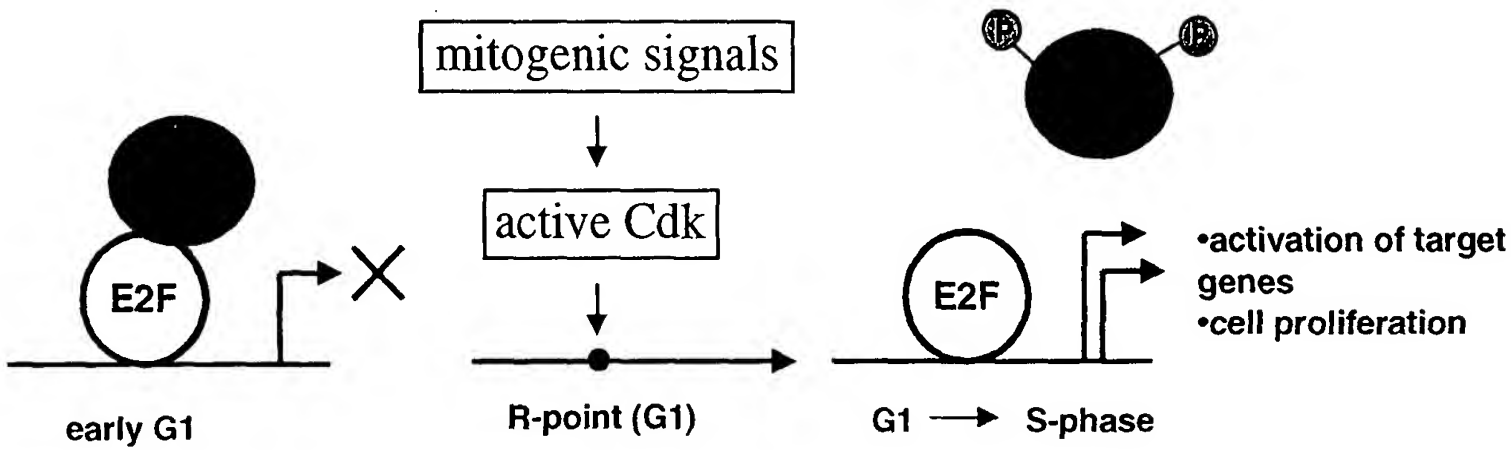
It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

Luminus Pty Ltd
~~BresaGen Limited~~
By its Registered Patent Attorneys
Freehills Patent Attorneys

AUSTRALIAN PATENT OFFICE SEC 113

24 September 1999

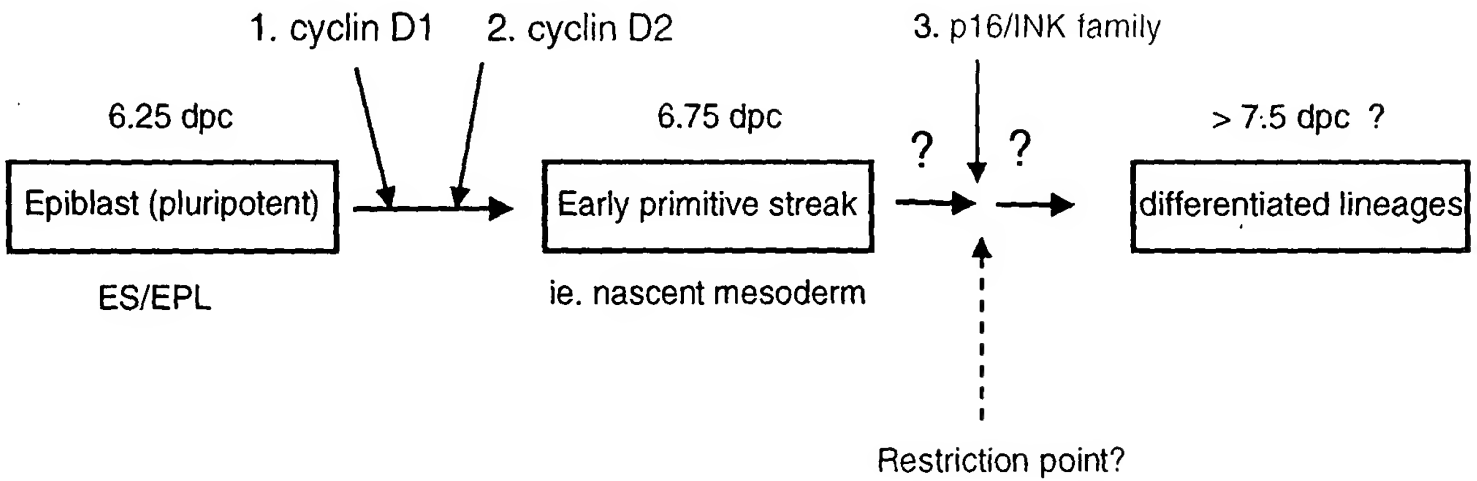


Restriction (R-) Point Control

Normal somatic cells	1. Regulated activation of pRb-kinases (Cdks) 2. pRb phosphorylation 3. Derepression of genes required for G1-S progression
Embryonic cells (tumour cells)	1. no regulated pRb kinase (Cdk2-cyclinE) 2. pRb constitutitively hyper-phosphorylated 3. E2F target genes constitutively active

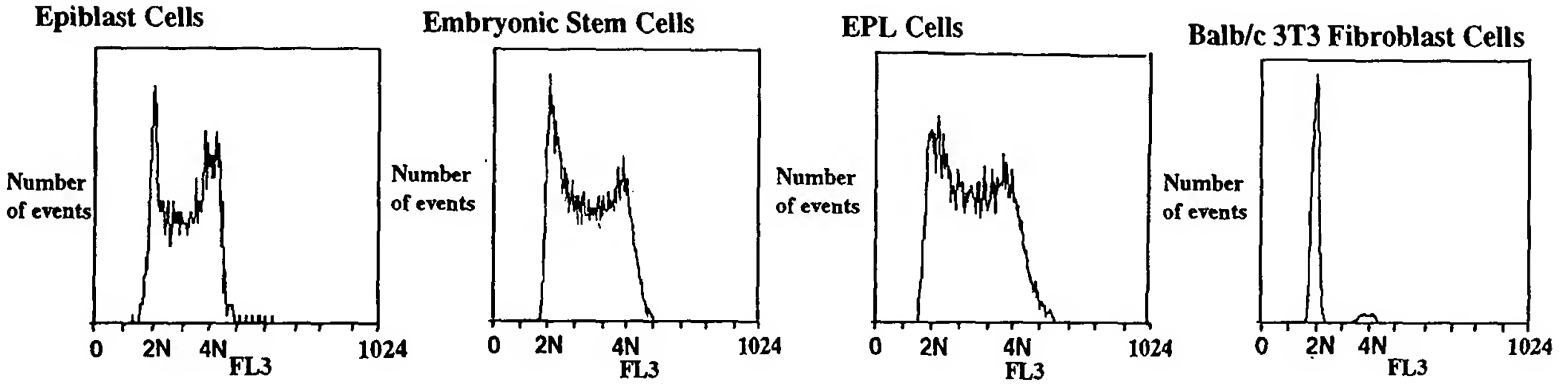
Figure 1

Remodelling of the cell cycle during embryonic development/differentiation



cell cycle length	8 hrs	4-6 hrs rapid proliferative phase	20-24 hrs

Figure 2


Epiblast Cells
Embryonic Stem Cells
EPL Cells
Balb/c 3T3 Fibroblast Cells
Number of events
0
2N
4N
1024
FL3

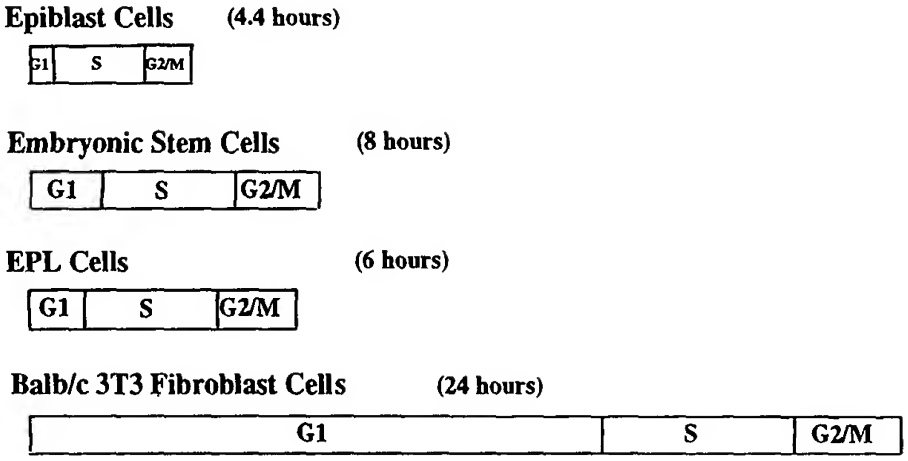
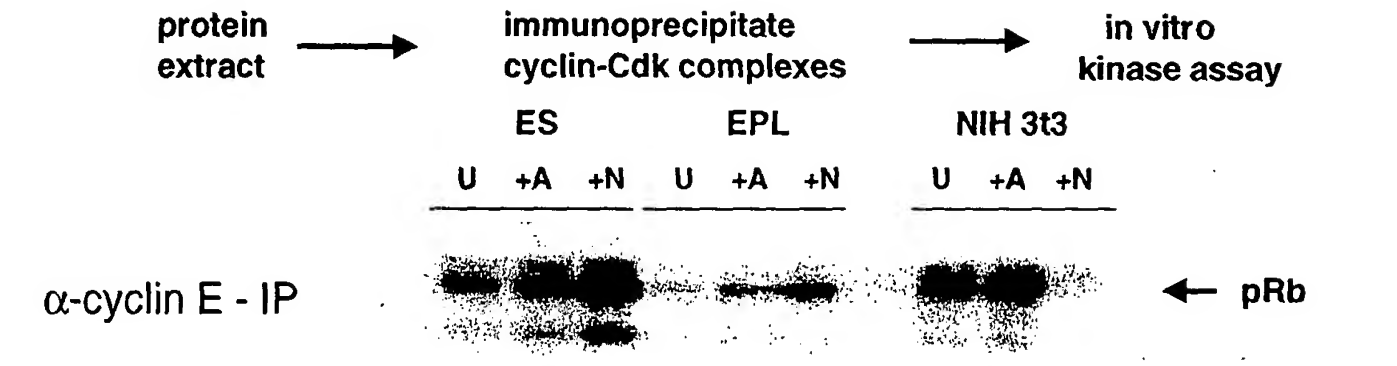

Epiblast Cells (4.4 hours)
G1
S
G2/M
Embryonic Stem Cells (8 hours)
G1
S
G2/M
EPL Cells (6 hours)
G1
S
G2/M
Balb/c 3T3 Fibroblast Cells (24 hours)
G1
S
G2/M

Figure 3

Unusual Cdk Regulation in Embryonic Stem Cells and Epiblast-Like (EPL) Cells

a)



b)

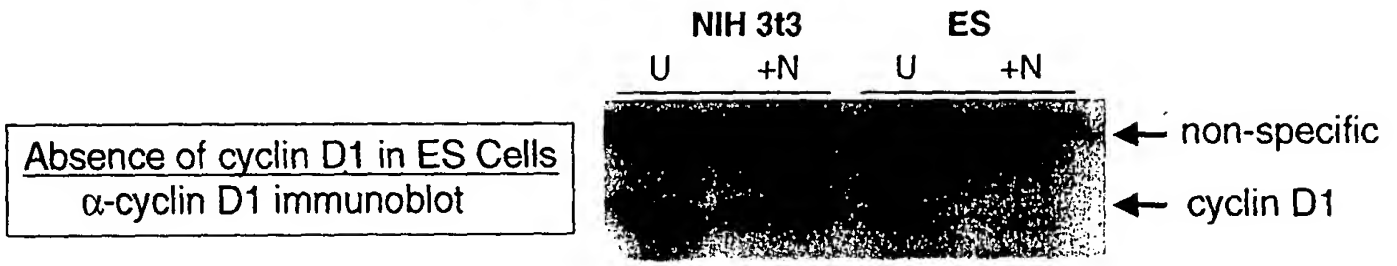


Figure 4

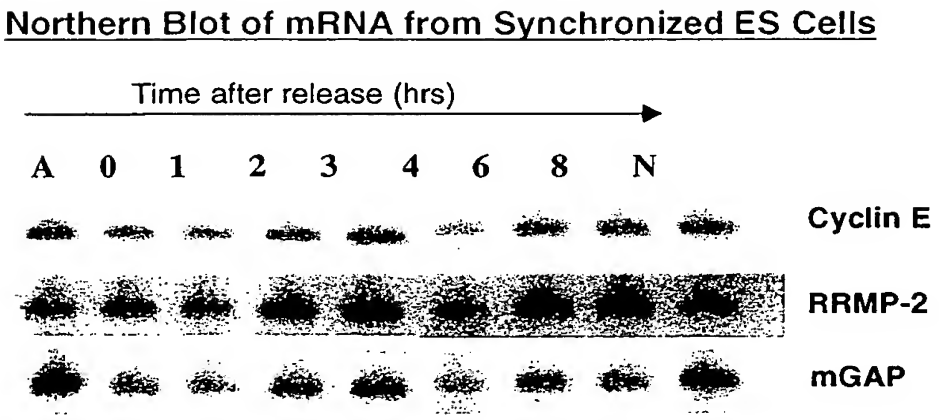

Northern Blot of mRNA from Synchronized ES Cells
Time after release (hrs)
A 0 1 2 3 4 6 8 N
Cyclin E
RRMP-2
mGAP

Figure 5

Western Analysis of Synchronised ES Cells

Cyclin E

Figure 6

Synchronization of ES cells

Unsynchronised ES cells

↓

Nocodazole (G2/M)

↓ Release

Aphidicolin (G1/S)

↓ Release

Sample collected

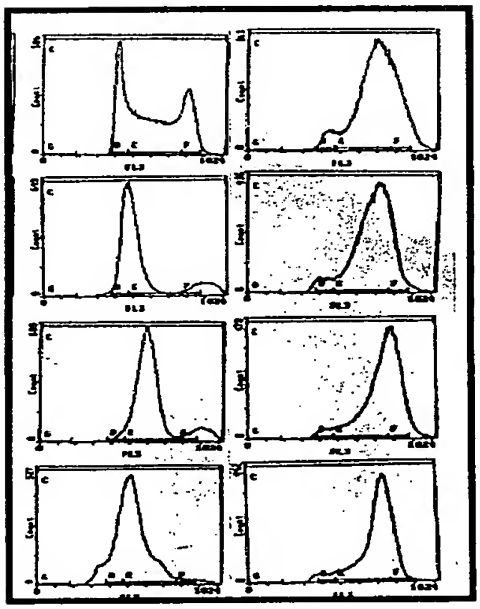


Figure 7